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Award Number: DAMD17-98-1-8608

TITLE: Therapeutic and Biologic Studies in a Murine Model of NF1

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REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20010327 090

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2000	3. REPORT TYPE AND DATES COVERED Annual (23 Sep 99 - 22 Sep 00)	
4. TITLE AND SUBTITLE Therapeutic and Biologic Studies in a Murine Model of NF1			5. FUNDING NUMBERS DAMD17-98-1-8608	
6. AUTHOR(S) Kevin Shannon, M.D.			8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of California San Francisco, California 94143-0962 E-MAIL: kevins@itsa.ucsf.edu				
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) This report describes progress made during the second year of support for a translational research project involving <i>Nf1</i> mutant mice with myeloid leukemia. Progress made during the first year is also reviewed to provide essential background. This study has two Technical Objectives. First, examining the therapeutic efficacy of two agents (1) mycophenolate mofetiel (MM) and, (2) a fusion toxin that targets the GM-CSF receptor. These compounds represent rational new approaches for treating NF1-associated tumors. MM has been tested in the mouse model and our preliminary data indicate that it is unlikely to provide benefit to NF1 patients. We are continuing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. We produced and purified a GM-CSF immunotoxin during year 1 and have tested this <i>in vitro</i> . These studies surprisingly revealed agonist (rather than inhibitory effects) of the conjugate, and we have been working to discern the basis for this.. In aim 2, we are utilizing <i>Nf1</i> mice to extend clinical observations suggesting that individuals with NF1 are susceptible to the development of therapy-associated second cancers. These studies were initiated in year 1 and are progressing well.. We are collecting tissues from mice with leukemia and will perform other correlative molecular studies over the next year. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.				
14. SUBJECT TERMS Neurofibromatosis, cancer			15. NUMBER OF PAGES 33	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Birnbaum, et al.

INTRODUCTION

Individuals with neurofibromatosis type 1 (NF1) are predisposed to specific benign and malignant neoplasms including juvenile myelomonocytic leukemia (JMML). Clinical data also suggest that children with NF1 have an increased risk of developing leukemia as a complication of genotoxic therapies for another primary cancer (Maris et al., 1997). Genetic and biochemical studies of patient leukemias performed in our laboratories have shown that *NF1* functions as a tumor suppressor gene in immature hematopoietic cells by negatively regulating the Ras signaling pathway (Bollag et al., 1996; Kalra et al., 1994; Miles et al., 1996; Shannon et al., 1994; Side et al., 1997). Similarly, heterozygous *Nf1* mutant (*Nf1*^{+/-}) mice show an increased incidence of myeloid leukemia and other cancers (Jacks et al., 1994). We found that treatment with the alkylating agent cyclophosphamide cooperates strongly with heterozygous inactivation of *Nf1* in murine leukemogenesis (Mahgoub et al., 1999). Homozygous *Nf1* mutant embryos (*Nf1*^{-/-}) die *in utero*. Like human JMML cells, *Nf1*^{-/-} fetal hematopoietic cells display a selective pattern of hypersensitivity to the cytokine growth factor GM-CSF in myeloid progenitor colony assays (Bollag et al., 1996; Largaespada et al., 1996). Adoptive transfer of these cells consistently induces a myeloproliferative disorder (MPD) that resembles JMML in irradiated recipients (Largaespada et al., 1996; Zhang, 1998). The predictable nature of this syndrome, the fact that transplanted mice survive for many months, and the well-characterized biochemical alterations in *Nf1*-deficient hematopoietic cells make this model attractive for testing novel therapeutics and for biologic studies of growth control.

The approved Statement of Work for this translational research project has two Technical Objectives which we are pursuing through two Specific Aims. Aim 1 proposes preclinical studies in recipient mice that have been transplanted with *Nf1*-deficient fetal liver cells to investigate the therapeutic efficacy of two agents (1) an inhibitor of *de novo* guanine nucleotide synthesis and, (2) a recombinant fusion toxin that targets the GM-CSF receptor. These compounds were chosen because they represent rational new approaches for treating NF1-associated tumors. We are also performing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. In aim 2, we are utilizing *Nf1* mice to extend clinical observations suggesting that individuals with inactivation of one *NF1* allele are susceptible to the development of therapy-associated second cancers. We are exposing cohorts of wild type and *Nf1*^{+/-} mice to either radiation therapy alone, or to radiation combined with cyclophosphamide to test the hypothesis that these mutagens will cooperate with each other and with inactivation of

Nf1 in tumorigenesis. We will examine tumor tissues for loss of heterozygosity (LOH) at *Nf1* and will perform other correlative molecular studies. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.

BODY

Technical Objective (Aim) 1: Testing Rational Therapeutics in Nf1 Mice

Overview of Preclinical Therapeutic Studies. This component involves independently testing the efficacy of two rational therapeutic agents to inhibit the growth of *Nf1*^{-/-} hematopoietic cells *in vivo*, and performing correlative biochemical and cell biologic assays. Our progress to date is presented below.

Preclinical Evaluation of Mycophenolate Mofetil (MM). As described in our proposal, MM is an inhibitor of the enzyme inosine 5' monophosphate dehydrogenase (IMPDH), which is required for *de novo* guanine nucleotide synthesis. The rationale for investigating this agent is based upon the idea that therapeutics that lower the ratio of GTP to GDP in the cell should decrease Ras-dependent growth because the activation state of Ras depends on the selective binding of GTP. The murine transplant model is ideal for initial studies that examine the efficacy of GTP reduction in inhibiting the abnormal growth of *Nf1*-deficient cells because the biochemical consequences of gene inactivation are well characterized in hematopoietic cells and because these cells rely heavily on the *de novo* pathway for nucleotide biosynthesis. Indeed, MM has shown anti-tumor efficacy in a number of preclinical studies performed in athymic nude mice. Additional background information, including calculations which suggested that cells in which an oncogenic *RAS* mutation or loss of *Nf1* would show enhanced sensitivity to a reduction in intracellular GTP concentrations, are presented in our proposal.

Summary of Progress in Year 1. During year 1, we determined that 400 mg/kg was a tolerable dose in wild-type mice and we initiated studies in irradiated recipients engrafted with either *Nf1*^{+/+} or *Nf1*^{-/-} fetal liver cells. These studies surprisingly showed that recipients of fetal liver cells developed dramatic increases in total leukocyte and myeloid cell counts, which were more pronounced in mice engrafted with *Nf1*^{-/-} cells.

Progress in Year 2. During this fund year, we extended the studies initiated in year 1. The results presented here represent combined data from years 1 and 2 of support. Leukocytosis has been a consistent finding

in mice treated with MM, with some *Nfl*^{-/-} recipients showing white blood cell counts in excess of 100,000 per mm³ (Figure 1). Bone marrow smears revealed striking myeloid hyperplasia. Spleen weights were greater in wild type recipients that received MM than in untreated controls, but not in *Nfl*^{-/-} recipients (Figure 2).

We performed additional cell biologic and biochemical studies to further elucidate the basis of this unexpected response to MM. Assays of bone marrow colony forming unit granulocyte macrophage (CFU-GM) numbers revealed a slight rise in splenic CFU-GM numbers from abnormally high baseline levels in a *Nfl*^{-/-} recipients; however, wild type mice showed a highly significant increase. These data were presented in our Progress Report from year 1. Thus, accelerated myelopoiesis in MM-treated wild type mice is associated with the appearance of substantial numbers of splenic CFU-GM. A major research goal for year 2 involved determining if we have succeeded in reducing GTP levels in primary cells and, if this is true, how this has altered Ras activation in resting and in growth factor-stimulated cells. We have succeeded in adapting published methods to measure cellular GDP and GTP concentrations *in vitro*. However, we have made multiple unsuccessful attempts to reliably quantify cellular levels of GTP and GDP in primary murine hematopoietic cells. It should be noted that there are no established methods in this area, and that performing signaling biochemistry on primary cells is challenging.

Plan for the Next Year of Funding. Our *in vivo* data preclude the therapeutic use of MM this agent in JMML. We will devote additional effort to correlating biochemical and clinical effects of MM in our model over the next few months. If we are unable to accurately measure GTP and GDP levels in primary cells, we will complete the other signaling experiments and will submit a paper describing our results.

Preclinical Evaluation of a DT_αGM-CSF Recombinant Fusion Toxin.

Investigations of myeloid progenitor colony growth in JMML patients and *Nfl*^{-/-} mice have implicated hypersensitivity to GM-CSF in leukemogenesis. In studies supported by NIH grant RO1 CA72614, we crossed lines of *Nfl* and *Gmcsf* mutant mice, and transferred doubly mutant fetal liver cells into irradiated wild type or *Gmcsf*-deficient recipients. Genetic ablation of GM-CSF production in host bone marrow and in donor fetal liver cells markedly attenuated the JMML-like MPD. Furthermore, adoptive transfer of doubly mutant bone marrow cells with established disease into secondary *Gmcsf*^{-/-} recipients reversed the MPD, which could be reinduced by treatment with exogenous recombinant murine GM-CSF. These data, which we reported in a paper that is included

in the Appendix (Birnbaum et al., 2000) implicate GM-CSF hypersensitivity as playing a central role in JMML. Recent experiments in which we crossed *Nf1* mutant mice with a line that has inactivated the β chain of the GM-CSF receptor provide further support for this hypothesis (data not shown). These studies suggest that inhibiting this signaling pathway might provide therapeutic benefit.

Summary of Progress in Year 1. A major goal of Technical Objective 1 involves the production of a highly purified recombinant DT_{Ct}GM-CSF fusion toxin and performing *in vitro* and *in vivo* murine studies in the *Nf1* mouse model system with the goal of specifically delivering the diphtheria toxin to myeloid cells via the GM-CSF receptor. Given emerging evidence suggesting that aberrant expression of growth factor receptors may underlie other pathogenic complications of NF1 (DeClue et al., 2000), this general therapeutic approach has broad implications. As summarized in our previous Progress Report, Dr. Perentesis produced and purified substantial amounts of the murine DT_{Ct}GM-CSF fusion toxin for *in vivo* murine studies. The initial *in vitro* characterization of this molecule revealed high level production and >95% purity of a monomeric protein with a molecular mass of ~57 kDa, the expected molecular mass of DT_{Ct}GM-CSF as deduced from its nucleic acid sequence. The final product of TSK-gel G2000 purification is estimated to exceed 98% purity. The integrity of expression of both the diphtheria toxin and GM-CSF moieties of DT_{Ct}GM-CSF was confirmed in immunoblot analysis employing antisera to diphtheria toxin.

In Vitro Effects of DT_{Ct}GM-CSF on Hematopoietic Cells. We performed a series of experiments over the past year to test the ability of this agent to inhibit the growth of cultured cell lines and of primary murine bone marrow cells. WEHI-3 cells were selected for the first series of studies because this cell line because we have previously shown that this cell line expresses surface GM-CSF receptors and dramatically up-regulated MAP kinase activity in response to GM-CSF (data not shown). Surprisingly, the DT_{Ct}GM-CSF had no demonstrable effects on the growth of WEHI-3 cells under a number of culture conditions (data not shown). To further address the biologic activity of the fusion toxin, primary mouse bone marrow cells were plated in methylcellulose cultures containing various concentrations of GM-CSF and DT_{Ct}GM-CSF immunotoxin. The results of these experiments are summarized in Figure 3. We observed no inhibitory effect of DT_{Ct}GM-CSF on CFU-GM colony formation. Indeed, the immunotoxin demonstrated significant agonist activity; that is, DT_{Ct}GM-

CSF induced colony formation in a dose-dependent manner (Figure 3). These results, which were confirmed with multiple lots of DT_{ct}GM-CSF, are consistent with the ability of the GM-CSF moiety of the recombinant fusion protein to recognize and bind the GM-CSF receptor. However, we did not detect the cytotoxic effects that we had expected from targeting diphtheria toxin to the GM-CSF receptor. Based upon these initial results, we have instituted major refinements in the expression and purification method for DT-mGMCSF to overcome problems with product stability and activity, as detailed below. These activities have included extensive *in vitro* biochemical and functional characterization of DT-mGMCSF.

Modifications and Refinements to Purification Methods for Recombinant Fusion Toxin DT-mGMCSF (Figure 4). Initial purification algorithms have demonstrated that it is feasible to produce substantial amounts of highly purified endotoxin-free recombinant murine DT-mGMCSF fusion toxin. As described above, the fusion toxin produced under these conditions possessed suboptimal activity, which is likely due to limited stability of the product. We have initiated a series of studies to modify the production and purification methods to enhance the yield and stability of the final product. Data demonstrating enhanced specific activity of the fusion product produced under these conditions is detailed below. The refined methods are detailed in Figure 4, and include several modification of diafiltration/ultrafiltration techniques, and elimination of inefficient chromatographic separation techniques. For the refinement of production methods, all manipulations of *E. coli* bearing intact recombinant fusion toxin were performed under modified Biosafety Level 3 (BL3) containment practices. In summary, *E. coli* HMS174(de3)plysS is transformed with pET11d:DT-mGMCSF and grown at 37°C in LB medium with carbenicillin (50 µg/ml) to an absorbance (Å595) of 0.55-0.65. The entire DNA sequence of the expression cassette of pET11d:DT-mGMCSF was determined in our laboratory and found to be correct. Expression of the fusion gene is induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells are then collected by centrifugation after one hour of induction. The bacterial pellets are resuspended in TE buffer (50mM Tris/20mM EDTA/100mMNaCl pH 7.8). Lysis of the cells is achieved by adding 5mg/ml of lysozyme and incubating 30 min in 40°C. The insoluble extract containing is resuspended in Triton-X buffer (89%TE buffer/11%vol/vol Triton-X) and homogenized briefly with tissumizer. After incubating at room temperature for 1 hour, the inclusion bodies are obtained by ultracentrifuge at 24,000g g for 50 min and solubilized in solubilization buffer (7M guanidine/0.1M Tris pH:8/2mM EDTA/65M dithioerythritol) overnight at room temperature. The solubilized protein

was collected by ultracentrifuge at 40,000g for 10 min, then diluted 100 times in refolding buffer (0.1M Tris pH:8/0.5M L-arginine/0.9mM Oxidized glutathione/2mM EDTA/0.1M Urea) for 48 hours at 100 °C. Procedure modifications included diafiltration and ultrafiltration of the refolded protein with trial buffers including: (a) 50mM NaCl/20mM Tris pH:7.8/100mM Urea, or (b) 50mM NaCl/20mM Tris pH:7.8. The product is then loaded on to Q-sepharose column and eluted with 0.3M NaCl in 20mM Tris pH:7.8. Other modifications include elimination of a redundant and inefficient second Q-sepharose chromatography. Final size-exclusion purification is conducted using a TSK-gel G2000 column.

Further Characterization of Recombinant DT-mGMCSF. The structure and integrity of the purified fusion toxin was characterized by standard methods using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. We also have enhanced our in vitro characterization methodologies to include functional assays of the catalytic activity of the fusion toxin (ADP-ribosyltransferase activity). This functional assay has confirmed that the refinements in the purification scheme result in a fusion toxin with significantly higher specific activity (Figure 5).

Western blot analyses using equine diphtheria antitoxin (anti-DT; Bethyl) antibodies and anti-mouse GMCSF antibodies were performed by standard methods²⁹ using 10% gels in a Mini-Protean II gel apparatus (Bio-Rad). Diphtheria toxin standards were obtained from Sigma. Primary antibodies were used at a dilution of 1:5000. Secondary antibodies, rabbit anti goat(Bethyl laboratories), covalently linked to horseradish peroxidase were used at a 1:5,000 dilution. We analyzed production lots of the recombinant murine DT-mGMCSF fusion toxin that had purified through the refined methods described above. SDS-polyacrylamide gel analysis of Q-sepharose chromatography column fractions revealed high level production and >95% purity of a monomeric protein with a molecular mass of ~57 kDa, the expected molecular mass of DT-mGMCSF as deduced from its nucleic acid sequence. The integrity of expression of both the diphtheria toxin and GMCSF moieties of DT-mGMCSF was confirmed in immunoblot analysis employing antisera to diphtheria toxin (Figure 6A) and murine GMCSF (Figure 6B). These results indicate high fidelity production of intact full-length recombinant murine DT-mGMCSF fusion toxin using this refined method. Importantly, no significant degradation is detected, reflecting high stability of the protein.

ADP-ribosyltransferase catalytic activity of the recombinant toxin is determined by measuring fusion toxin incorporation of [adenylate-³²P]NAD⁺ into the EF-2 of reticulocyte lysates, and comparison of activity

with a reference standard of native diphtheria toxin. Approximately 300 ng of DT_{ct}GMCSF is incubated at 37°C in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM dithiothreitol, 1 mM EDTA, and 2.5 μM [adenylate-³²P]NAD⁺ ($\approx 1 \times 10^6$ cpm). Reference reactions with standard amounts of native diphtheria toxin contain 200 ng of nicked diphtheria toxin is incubated at 37°C for 15 minutes in a 50 microliter reaction mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM dithiothreitol, 1 mM EDTA, and 2.5 μM [adenylate-³²P]NAD⁺ ($\approx 1 \times 10^6$ cpm). The reaction is terminated by adding 50 microliters of 20% (wt/vol) cold trichloroacetic acid. The insoluble protein is collected by vacuum filtration onto 0.45 micron nitrocellulose filters and twice rinsed with 5% (wt/vol) cold trichloroacetic acid. The filters are dried and placed in scintillation counter vials, and approximately 1 ml of 95% Econo-Safe scintillation fluid is added. Radioisotope incorporation into protein is measured in a Beckman LS7000 scintillation counter.

Figure 5 shows results of ADP-ribosyltransferase catalytic activity of the recombinant toxin after production with various methodologies, and comparison to native DT. These results show that methods employing diafiltration and ultrafiltration of the refolded protein with 50mM NaCl/20mM Tris pH:7.8 buffer and elimination of redundant Q-sepharose chromatography steps resulted in production of fusion toxins with the highest specific activity. The optimal preparations of fusion toxin have specific activity comparable with that of native diphtheria toxin.

In summary, comprehensive investigation during this project period have optimized the production of high specific activity murine DT_{ct}GMCSF, and include: (a) verification of entire DNA sequence of DT_{ct}GMCSF expression cassette, and (b) identification of optimal diafiltration/ultrafiltration techniques and optimized anion-exchange chromatography methods. Validation measures have included: (a) verification of high level production of intact full-length fusion toxin assessed by unequivocal immunoblot results using both anti-DT and anti-murine GMCSF antibodies, and (b) functional evaluation confirming high specific activity ADP-ribosyltransferase activity of the recombinant DT_{ct}GMCSF fusion toxin.

Plans for the Next Year of Funding. We plan to finish characterizing lots of DT-mGMCSF produced using our improved methodology. We will utilize the ability of DT-mGMCSF to inhibit CFU-GM colony formation in methylcellulose cultures as a primary test. After we demonstrate an inhibitory effect of DT-mGMCSF on normal murine bone marrow, we will perform competition experiments to test the prediction that high

concentrations of GM-CSF will antagonize the effects of the immunotoxin. We will also compare the effects of DT-mGMCSF on fetal liver cells of all three *Nf1* genotypes. Finally, we will test the specificity of DT-mGMCSF for the GM-CSF receptor by investigating the ability of the immunotoxin to inhibit the growth of bone marrow cells from mice with a mutation in the β common chain of the GM-CSF receptor (*GMR β*). We have generated a colony of these animals over the past year. If DT-mGMCSF is targeting the receptor, we predict that it will not inhibit the growth of these cells. Together, these *in vitro* experiments will provide valuable preliminary data regarding the potency and specificity of DT-mGMCSF.

When we have *in vitro* data establishing that DT-mGMCSF inhibits CFU-GM colony growth by targeting the GM-CSF receptor, we will perform the *in vivo* studies proposed in our application. In a pilot study, groups of 5 wild-type mice will receive total doses of 1 μ g, 2 μ g, 5 μ g, 10 μ g, or 20 μ g of murine DT_{ct}GMCSF administered as 5 daily i.p injections. These regimens were chosen because they were well tolerated and yielded very encouraging results in preliminary studies of human DT_{ct}GMCSF in mice injected with human leukemia cells. The dose-finding study will be expanded with either lower or higher dose ranges depending on the observed toxicities. In treatment regimens involving *Nf1* mice, the same numbers of *Nf1*^{-/-} and *Nf1*^{+/+} transplant recipients will be enrolled in phase 1 and phase 2 experiments and the clinical and laboratory observations will be identical. The phase 1 experiment will also involve treating a cohort of the homozygous *GMR β* mutant mice with DT_{ct}GMCSF. These mice provide an excellent model for testing the specificity of DT_{ct}GMCSF therapy, and we predict that they will show no changes in blood cell counts even at high doses of the recombinant fusion toxin.

Technical Objective (Aim) 2: Chemotherapy and Radiation Studies

Overview. These studies are based upon clinical observations suggesting that children with NF1 are at increased risk of developing myeloid and other tumors after being treated with multi-modal therapy for another cancer (Maris et al., 1997). These human data implicated exposure to alkylating agents in the development of therapy-related leukemia (t-ML). Based on these clinical findings and on the 10% risk of leukemia in untreated *Nf1*^{+/-} mice (Jacks et al., 1994), we exposed *Nf1*^{+/-} mice to mutagenic agents frequently used to treat malignancies in patients with and without NF1 (Mahgoub et al., 1999). We found that treating heterozygous *Nf1* mice with cyclophosphamide (CP), a commonly-used chemotherapeutic agent, markedly increased the incidence of myeloid malignancies and shortened the latency to disease onset. In this study, CP

treatment was associated with the development of a myeloid disorder in 16 of 37 *Nf1*^{+/-} mice, but in only 2 of 30 wild-type animals (Fig 2). Most affected mice developed MPD; this was frequently associated with loss of heterozygosity (LOH) involving the wild-type *Nf1* allele in 129/Sv mice but with a low incidence in the F1 129/Sv x C57BL/6 background. Cytogenetic analysis of bone marrow and spleen cells from mice with leukemia revealed a normal karyotype. To ascertain if LOH on Southern blots was associated with submicroscopic deletions of *Nf1* or with duplication of the mutant allele, we used a genomic *Nf1* probe from the disrupted segment of the gene to perform fluorescence *in situ* hybridization (FISH) analysis of hematopoietic cells from 3 mice. FISH revealed two structural copies of *Nf1* in each case, a result that is consistent with the pattern of allele loss in bone marrow cells from children with NF1 (K. Stephens, MM Le Beau, and KMS; unpublished data). The cytogenetic and FISH experiments were performed in collaboration with Dr. Michelle Le Beau (University of Chicago). Preliminary data from these studies were presented in our original application and a paper describing this study was provided with the Appendix to our last Progress Report (Mahgoub et al., 1999).

This *in vivo* model of t-ML has a number of novel features that facilitate basic and translational research studies of this important clinical disorder. First, the fact that *Nf1* mice recapitulate clinical observations made in NF1 patients suggest that this model will be highly relevant for understanding specific aspects human t-ML. Second, *Nf1* provides a genetic target to examine the mechanism(s) of alkylator-induced DNA damage in hematopoietic cells. Finally, this model allows us to undertake controlled experiments that are neither feasible nor ethical in humans. We are exploiting this system to ask if radiation therapy, alone and in combination with CP, accelerates tumorigenesis in heterozygous *Nf1* mice. This question is highly relevant to the care of individuals with NF1 because radiation therapy is used frequently to treat NF1-associated tumors. Indeed, our initial data led the Children's Cancer Group to modify the treatment of children with NF1 who develop brain tumors so that they are not assigned to alkylator-intensive regimens. In addition to treating *Nf1* mutant mice with radiation and CP, we are performing molecular analyses at the *Nf1* locus and are examining the incidence of hypoxanthine guanine phosphoribosyl transferase (*Hprt*) mutations as an *in vivo* measure of DNA damage.

Summary of Progress in Year 1. In year 1, we escalated the dose of CP and found that F1 C57BL/6 x 129/Sv mice tolerate a course of 200 mg/kg/week for 6 weeks, and consistently develop neutropenia. We next showed that we could administer a single 2 or 3 Gray (200 or 300 rads) dose of total body irradiation as a single fraction two weeks after the last dose of CP.

The use of total body irradiation insures that all of the blood-forming marrow is exposed, and previous data have shown that 2-3 Gy is more leukemogenic than higher or lower doses in susceptible mouse strains (Major and Mole, 1978). We found that *Nf1*+/- 129Sv x C57BL6 F1 mice tolerated these treatment regimens well with no deaths occurring during or after the radiation phase of the study. In these pilot studies, treatment was associated with a variable degree of leukopenia and anemia, depending on the regimen used (see below). Based on these results, we are comparing four groups of mice (1) CP + 3 cGy, (2) CP alone, (3) irradiation alone, and, (4) no treatment. In order to minimize the number of animals treated, the design included entering fewer mice in the control and CP alone arms (groups 1, 2, 5, and 6) because we have already ascertained the expected incidence of myeloid diseases in these cohorts. We began enrolling mice in July, 1999 and entered 42 by the end of year 1.

Enrollment and Characteristics of Study Mice. We bred, genotyped, and treated the remainder of the cohort between the end of year 1 and May 2000. A total of 189 C57BL6/129Sv mice have been enrolled and have been followed a median of 7.5 months (range 3.5 – 13 mons) from the end of the eight week treatment phase. The mice tolerated the treatment well, with only two presumed treatment related mortalities occurring approximately three weeks following completion of treatment with CP and irradiation. As expected from the pilot data presented in our last Progress Report, total white blood cell counts, absolute neutrophil counts, and hemoglobin levels declined in response to CP, and recovered following completion treatment (Figure 7). Administration of radiation delayed the onset hematologic recovery in CP-treated animals (Figure 7). Blood counts normalized by three weeks following completion of treatment.

Table 1
Characteristics of Mice Entered

Treatment Group	Target	Wild-type	<i>Nf1</i> +/-
None	15	17	14
CP only	15	18	14
Radiation	30	34	29
CP + Radiation	30	35	28

Nine mice have died to date, including two with solid tumors. One wild-type male mouse treated with CP + radiation developed a probable intrathoracic lymphoma approximately three months following completion

of CP + radiation, and a male heterozygous mouse treated with CP alone developed a probably sarcoma and was sacrificed 6 months following completion of treatment with CP.

Hprt Assay. Hypoxanthine guanine phosphoribosyl transferase (HPRT) is a cellular enzyme crucial in the metabolism of the chemotherapeutic agent thioguanine into deoxythioguanine triphosphate which can then be incorporated into DNA and cause cell death. Lymphocytes that have inactivated *Hprt* acquire the ability to proliferate in the presence of 6-thioguanine because they are unable to convert this drug to its active metabolite. *Hprt* mutation rate has been used as a surrogate marker for DNA damage induced by mutagenic compounds such as CP and irradiation, and for evaluating potential chemoprotective compounds (Kataoka et al., 1996; Meng et al., 1998). However, it is not known if reducing the frequency of *Hprt* inactivation will correlate with a decrease in the risk of therapy-related cancer *in vivo*. If this proves true, *Hprt* could be used as a surrogate marker to test the mutagenic potential of new chemotherapeutic agents and of specific regimens.

We established the *Hprt* assay in our laboratory using published methods (Meng et al., 1998). *Hprt* mutation frequency is measured in male mice because this locus is on the X chromosome. We initiated these studies in C57BL6/129Sv mice treated with CP at a dose of 200 mg/kg/week for either 1 week (single dose) or for 6 weeks. The mice are sacrificed and splenocytes are isolated 55-60 days after the last drug dose. Preliminary data from a cohort of mice treated for 1 week showed a greater than 10 fold increase in the mutation frequency of CP-treated mice compared to controls (mutation frequency = 4.7×10^{-5} in CP-treated mice vs. 3.3×10^{-6} in the controls; $p = 0.038$). In a study supported by the American Cancer, we are testing the chemopreventive agent amifostine in CP-treated *Nf1* mice. As shown in Figure 8, amifostine markedly reduced the *Hprt* mutation rate in mice that received a single dose of CP, but was associated with a paradoxical increase in mutation rates after a full 6 week course. Consistent with this, our preliminary data suggest that amifostine does not protect *Nf1* mice treated with CP for 6 weeks from leukemia. In experiments that are currently in progress, we are comparing *Hprt* mutation rates in control mice with the rates in mice treated with CP alone, CP + radiation, or radiation alone.

We have also successfully adapted the protein truncation method that we have used to detect *NF1* mutations in human leukemias to analyze the murine homolog. This assay will prove useful for analyzing tumor tissues from our cohort that do not show LOH for somatic mutations in the wild-type *Nf1* allele.

Plan for the Next Year of Funding. Having achieved our goal of enrolling and treating the entire cohort during year 2, we will continue to observe these mice and will begin collecting tissues and follow-up data. We expect that substantial numbers of leukemias and solid tumors will arise during the next 6-12 months. We are comparing *Hprt* mutation rates in untreated mice and in mice treated with CP alone, radiation alone, or CP + radiation to test the hypothesis that *Hprt* mutation rates will predict survival and cancer rates. We will also investigate any murine leukemias or solid tumors for LOH at *Nf1*, and will perform IVTT to define the incidence and nature of somatic *Nf1* mutations in samples that do not show LOH.

KEY RESEARCH ACCOMPLISHMENTS

- Developed breeding stocks of mouse strains and generated recipients repopulated with *Nf1*^{-/-} or wild-type fetal liver cells.
- Completed *in vivo* studies of MM in transplant recipients.
- Performed initial correlative biochemical studies of tissues from MM-treated mice.
- Produced DT_{ct}GM-CSF, and tested this compound *in vitro*.
- Devised improved process for producing and evaluating DT_{ct}GM-CSF
- Entered entire cohort of mice in study evaluating the interaction between radiation and CP in tumorigenesis, and have begun collecting tissues.
- Established *Hprt* and protein truncation assays.

REPORTABLE OUTCOMES

(a) Review Article

Weiss B, Bollag G, Shannon KM. Hyperactive Ras as a therapeutic target in neurofibromatosis type 1. *Am J Med Genet* 1999; 89: 14-22.

(b) Model Development

The studies conducted to date have established a regimen for administering cyclophosphamide with and without radiation to F1 C56BL6/129Sv mice that should be useful for future studies of tumorigenesis and chemopreventive strategies in *Nf1* mice. The *Hprt* and protein truncation assays are generally applicable for mutation detection studies in *Nf1* mice.

(c) Employment and Research Opportunities

Richard Chao, M.D. is a fellow in adult hematology/oncology who is supported by this award. Dr. Chao is primarily working on the radiation/cyclophosphamide studies. He is interested in pursuing a career in translational research.

Brian Weiss, M.D. is a fellow in pediatric hematology/oncology who is participating in the experimental therapeutics studies. His salary has been supported by a training fellowship from the Frank A. Campini Foundation, and he recently received a KO8 award from the National Cancer Institute.

Alfred Au, Zabi Wardak, Myla Sanchez, and Abigail Peterson are technical personnel in the investigator's laboratories who have received partial salary support from this award.

CONCLUSIONS

Our progress and plans for the duration of this award are presented in detail in the Body. The nature of translational research is that it involves considerable effort in generating reagents, performing the experiments, and obtaining long-term follow-up. These studies are proceeded well to date and are on or ahead of schedule. We have encountered some difficulties in measuring GDP and GTP levels in primary cells, and are working actively on the problem. We were also disappointed that the initial DT_{Ct}GM-CSF preparations targeted the GM-CSF receptor, but were not cytotoxic. We have improved the methods used to produce DT_{Ct}GM-CSF, and have validated our *in vitro* assays for testing this immunotoxin. The entire of mice has now been enrolled and treated with CP and/or radiation, and we are carefully following this cohort.

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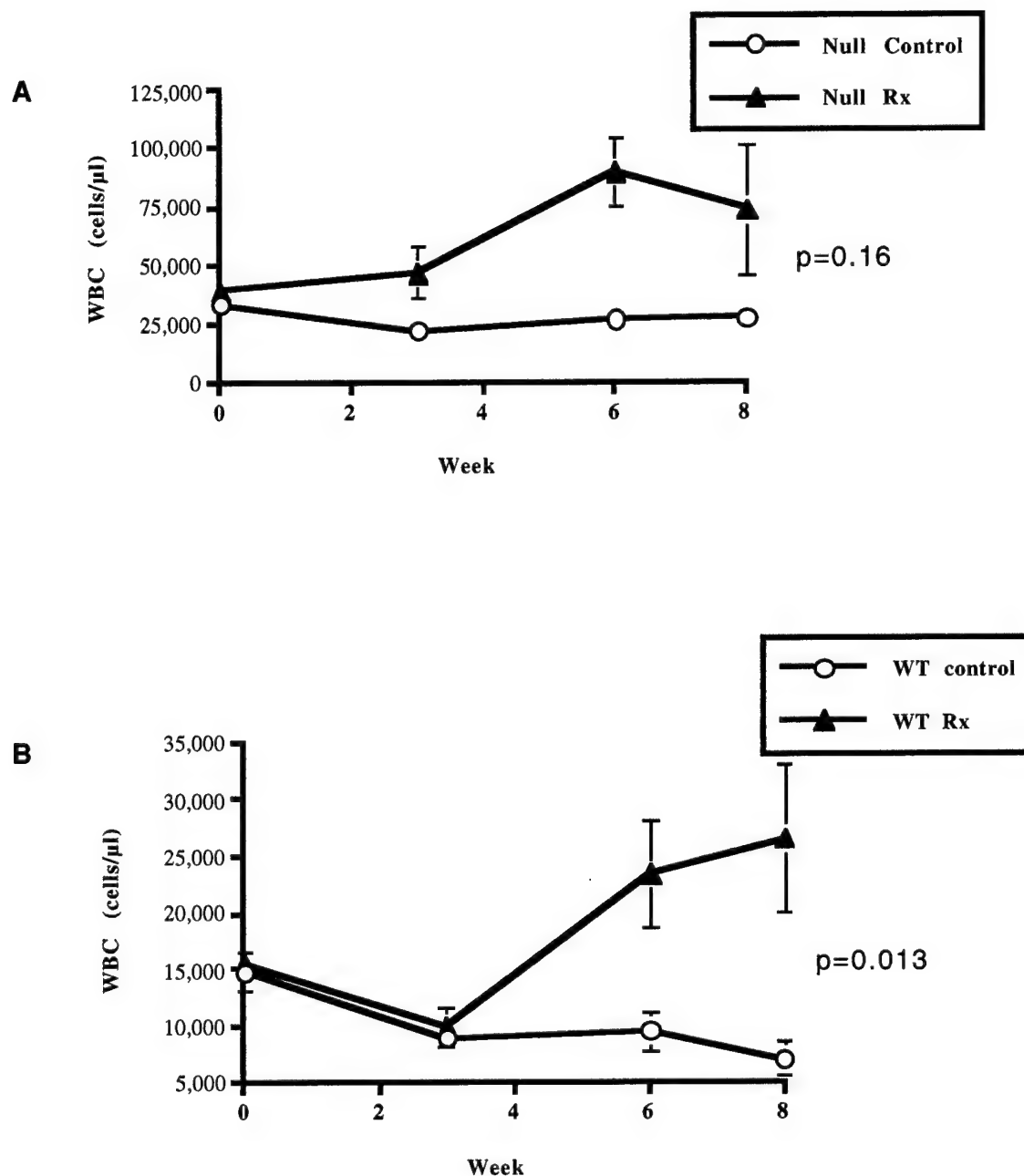


Figure 1. White blood cell counts in recipient mice engrafted with *Nf1*^{-/-} (panel A) or *Nf1*^{+/+} (panel B) fetal liver cells. Control mice were treated with sterile vehicle and “Rx” mice were treated with 400 mg/kg/day of MM daily for 8 weeks. Note that baseline white blood cell counts were much higher in recipients of *NF1*^{-/-} cells. The increase in WBC was due to a rise in myeloid cells in both groups of Rx mice.

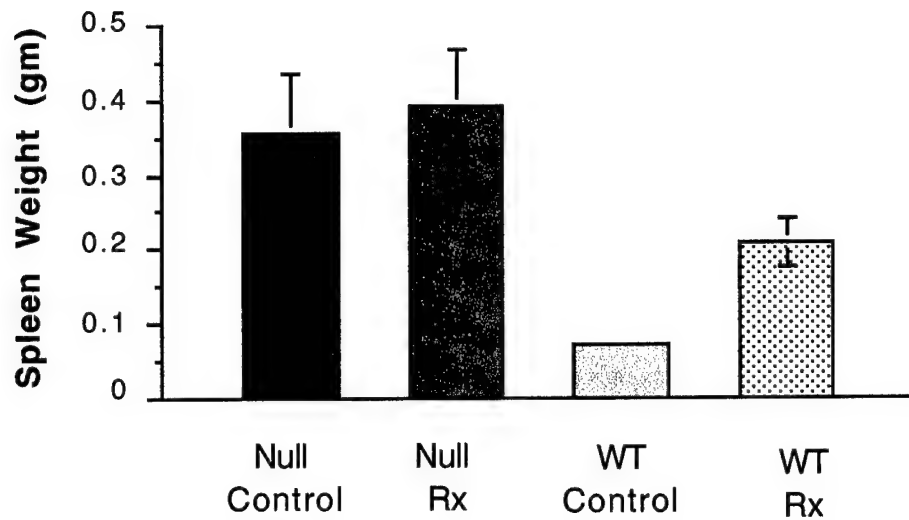


Figure 2. Spleen weights in recipient mice engrafted with *Nf1*^{-/-} or *Nf1*^{+/+} fetal liver cells. Control mice were treated with sterile vehicle and “Rx” mice were treated with 400 mg/kg/day of MM daily for 8 weeks. *Nf1*^{-/-} recipients treated with MM did not have a significantly larger spleens than *Nf1*^{-/-} controls ($p=0.8$). On the other hand, *Nf1*^{+/+} recipients exposed to MM had significantly larger spleens than *Nf1*^{+/+} controls ($p<0.05$), and in fact had a mean splenic weight which approached that of MM-treated *Nf1*^{-/-} mice ($p=0.9$).

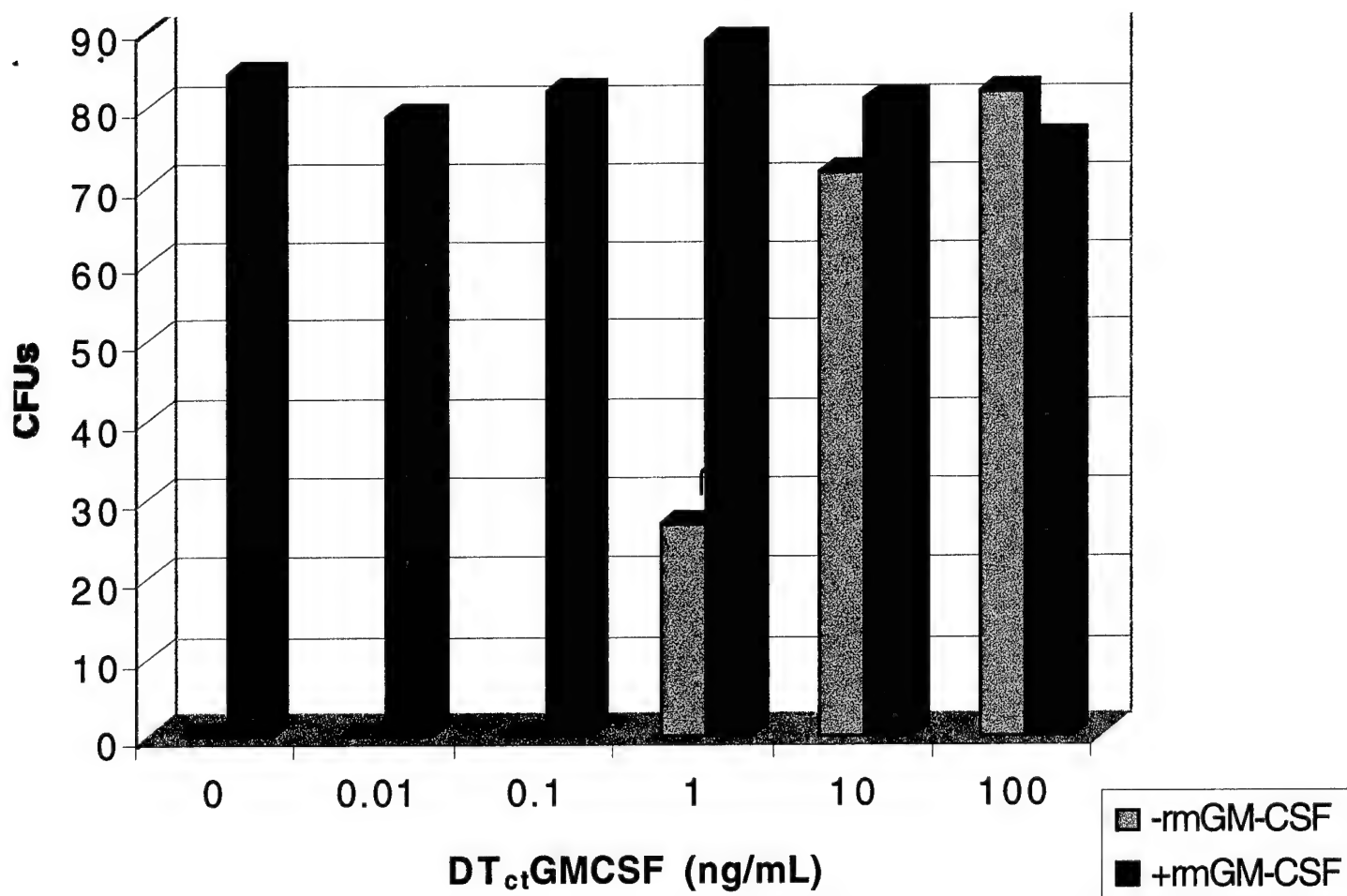


Figure 3. Effects of DT_{ct}GM-CSF on CFU-GM Colony Growth from Normal Mouse Bone Marrow. Marrow mononuclear cells were plated in methylcellulose medium containing various concentrations of DT_{ct}GM-CSF in the presence or absence of GM-CSF as shown. DT_{ct}GM-CSF did not antagonize colony growth at any concentration tested, and showed agonist activity at 1 ng/mL and above.

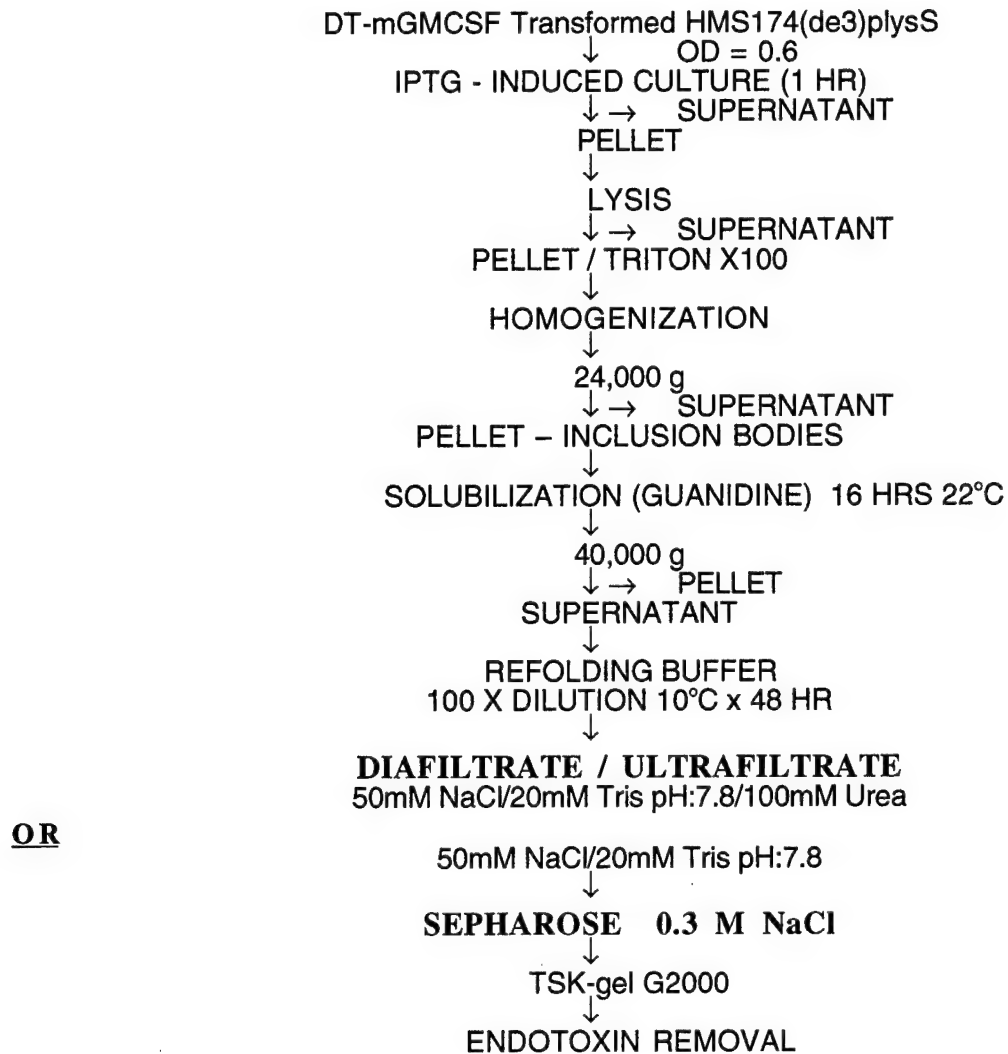
DT-mGMCSF EXPRESSION AND PURIFICATION

Figure 4. Expression and purification of the recombinant DT-mGMCSF fusion toxin. Refinements in technique indicated by underscore (see text for details).

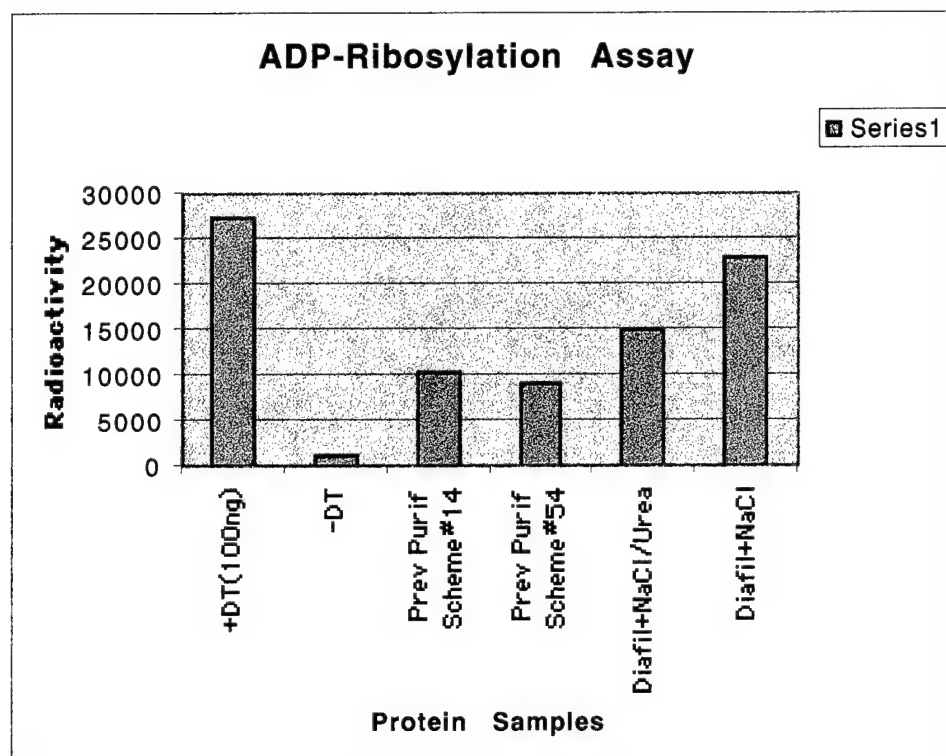


Figure 5. Measurement of specific functional activity of recombinant fusion toxin preparations by ADP-ribosylation assay. Legend: DT (native diphtheria toxin); Pre-Purification Scheme (samples of murine DT-mGMCSF produced prior to optimizations and refinements in production and purification methods); Diafil+NaCl/Urea (recombinant fusion toxin production runs using 50mM NaCl/20mM Tris pH:7.8/100mM Urea, and subsequent modified Q-Sepharose chromatography); Diafil+NaCl (recombinant fusion toxin production runs using 50mM NaCl/20mM Tris pH:7.8, and subsequent modified Q-Sepharose chromatography). Optimal specific activity is observed in fusion toxin produced with 50mM NaCl/20mM Tris pH:7.8, and subsequent modified Q-Sepharose chromatography.

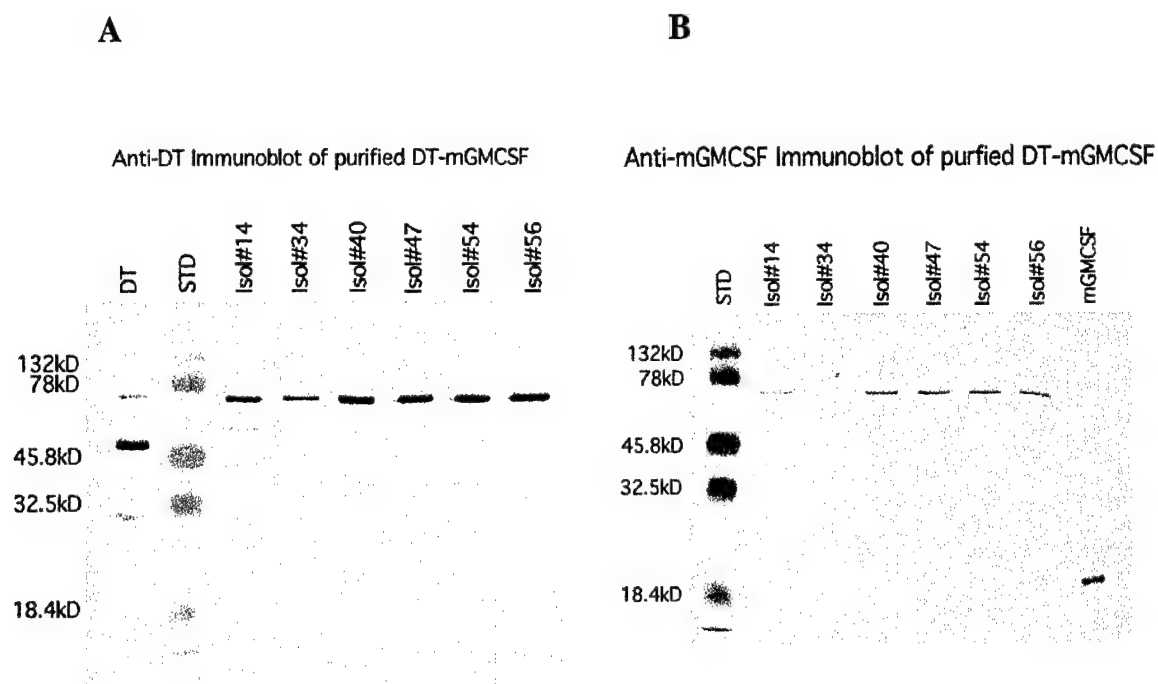


Figure 6. Immunoblots of purified recombinant DT-mGMCSF fusion toxin after implementation of refinements in diafiltration/ultrafiltration and chromatography methods (see text for details). Legend: DT (native diphtheria toxin); STD (molecular weight standards); Iso#14, Iso#34, Iso#40 (recombinant fusion toxin production runs using 50mM NaCl/20mM Tris pH:7.8/100mM Urea, and subsequent modified Q-Sepharose chromatography); Iso#47, Iso#54, Iso#56 (recombinant fusion toxin production runs using 50mM NaCl/20mM Tris pH:7.8, and subsequent modified Q-Sepharose chromatography). High production intact fusion toxin is shown with both methods.

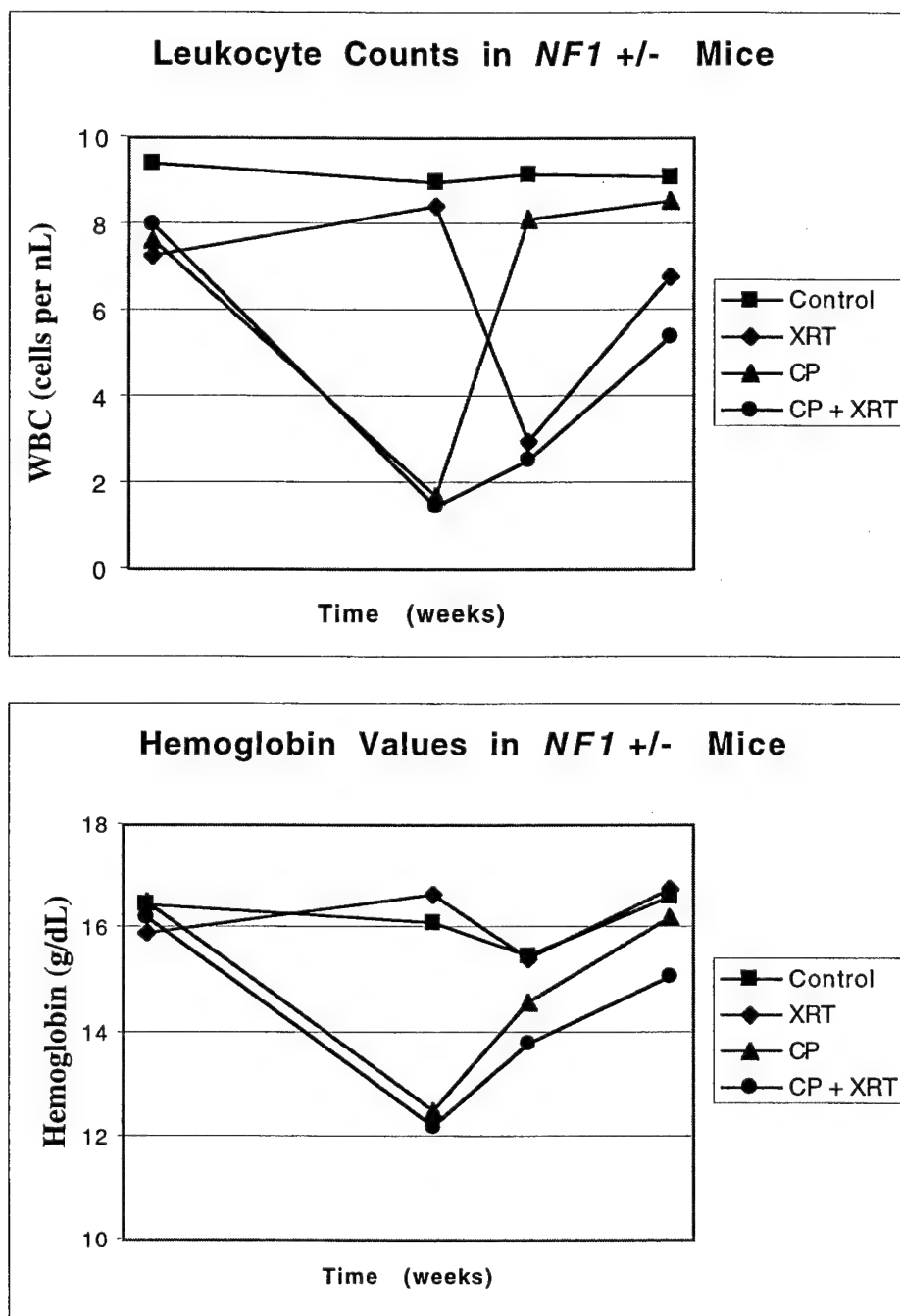


Fig. 7. Heterozygous *NF1* mutant mice were either untreated (Control) or treated with 3 Gy total body irradiation (XRT), 6 weekly intraperitoneal injections of 200 mg/kg of cyclophosphamide (CP), or the combination of cyclophosphamide and radiation (CP + XRT). Blood counts were taken before treatment, after completion of CP, after the completion of radiation, and three weeks following radiation. Changes in leukocyte counts and hemoglobin are shown.

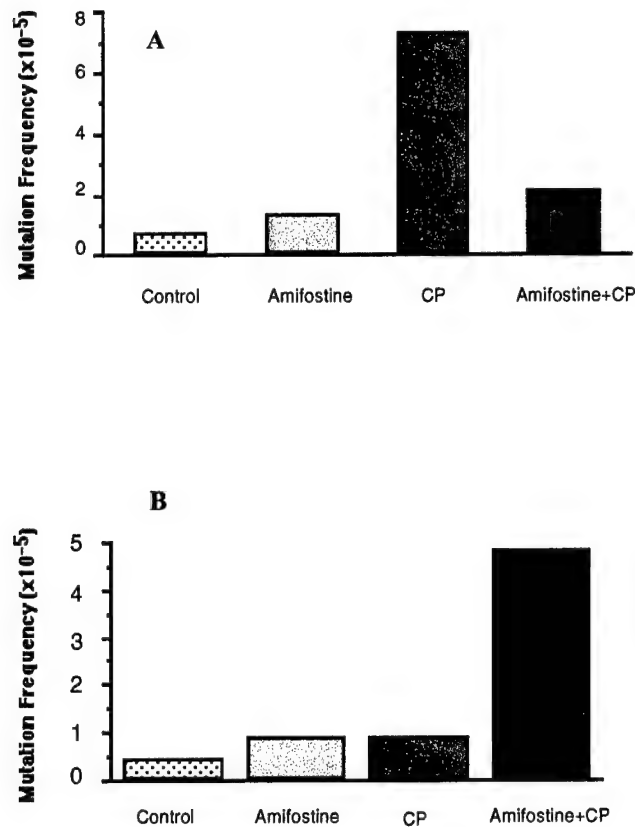


Figure 8. F1 129/Sv x C57Bl6 *Nf1*^{+/-} mice ages 2-4 months received either a single dose (A) or 6 weekly doses (B) of amifostine at 200 mg/kg or control (sterile saline) by intraperitoneal injection 30 minutes prior to being injected intraperitoneally with either CP at 200 mg/kg or sterile water. Mice were sacrificed at 55-60 days and splenocytes were harvested. In mice treated for one week (A), control treated mice had a *Hprt* mutation frequency of 7×10^{-6} compared to 7×10^{-5} in CP-treated mice. Mice treated with amifostine + CP had a mutation frequency of 2×10^{-5} , approximately 70% less than mice treated with CP alone. In mice treated for 6 weeks (B), control treated mice had a *Hprt* mutation frequency of 4×10^{-6} compared to approximately 9×10^{-6} in CP-treated and amifostine-treated mice. Mice treated with amifostine + CP had a mutation frequency of almost 5×10^{-5} , 5 times that of CP alone.

Nf1 and *Gmcsf* Interact in Myeloid Leukemogenesis

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Summary

The *NF1* tumor suppressor gene encodes neurofibromin, a GTPase-activating protein (GAP) for p21^{ras} (Ras). Children with *NF1* are predisposed to juvenile myelomonocytic leukemia (JMML). Some heterozygous *Nf1* mutant mice develop a similar myeloproliferative disorder (MPD), and adoptive transfer of *Nf1*-deficient fetal liver cells consistently induces this MPD. Human JMML and murine *Nf1*-deficient cells are hypersensitive to granulocyte-macrophage colony-stimulating factor (GM-CSF) in methylcellulose cultures. We generated hematopoietic cells deficient in both *Nf1* and *Gmcsf* to test whether GM-CSF is required to drive excessive proliferation of *Nf1*^{-/-} cells in vivo. Here we show that GM-CSF plays a central role in establishing and maintaining the MPD and that recipients engrafted with *Nf1*^{-/-} *Gmcsf*^{-/-} hematopoietic cells are hypersensitive to exogenous GM-CSF.

Introduction

Ras proteins modulate diverse cellular processes by cycling between an inactive guanosine diphosphate (GDP)-bound state (Ras-GDP) and an active guanosine triphosphate (GTP)-bound state (Ras-GTP) (reviewed in Bourne et al., 1990; Wittinghofer, 1998). *RAS* mutations are found in many human cancers, including myeloid leukemias (reviewed in Bos, 1989; Rodenhuis, 1992). Mutant *RAS* alleles encode proteins that accumulate in the GTP-bound conformation because of defective GTP hydrolysis (reviewed in Bourne et al., 1990; Downward, 1990; Hall, 1992; Wittinghofer, 1998). GTPase-activating

proteins (GAPs) negatively regulate Ras output by accelerating the slow intrinsic Ras GTPase activity (reviewed in Boguski and McCormick, 1993; Bernards, 1995). The neurofibromatosis type 1 (*NF1*) tumor suppressor gene encodes neurofibromin, which functions as a GAP for Ras (Boguski and McCormick, 1993; Bernards, 1995).

Individuals with neurofibromatosis type 1 (*NF1*) are predisposed to specific cancers, including fibrosarcoma, pheochromocytoma, astrocytoma, and juvenile myelomonocytic leukemia (JMML) (reviewed in Side and Shannon, 1998). JMML is a relentless myeloproliferative disorder (MPD) of young children characterized by overproduction of myeloid lineage cells that infiltrate hematopoietic and nonhematopoietic tissues (reviewed in Emanuel et al., 1996; Arico et al., 1997). JMML cells from children with *NF1* frequently delete the normal *NF1* allele, and this is associated with biochemical evidence of hyperactive Ras signaling (Shannon et al., 1994; Bollag et al., 1996; Side et al., 1997). Furthermore, oncogenic *RAS* mutations are detected in the bone marrows of 20%–30% of children with JMML who do not have *NF1* but are absent in patients with *NF1* (Kalra et al., 1994). Approximately 10% of heterozygous *Nf1* mutant mice (*Nf1*^{+/-}) spontaneously develop a JMML-like MPD with somatic loss of the wild-type *Nf1* allele in bone marrow cells (Jacks et al., 1994). Homozygous *Nf1*-deficient embryos (*Nf1*^{-/-}) die in utero between E12 and E14 from cardiac defects (Brannan et al., 1994; Jacks et al., 1994); however, adoptive transfer of *Nf1*-deficient fetal liver cells consistently induces in irradiated recipient mice MPD that is associated with hyperactive Ras (Largaespada et al., 1996; Zhang et al., 1998). Together, these human and murine data provide strong evidence that the tumor suppressor function of *NF1* in myeloid lineage cells is mediated through the ability of neurofibromin to negatively regulate Ras output.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) binds to receptors expressed on a subset of myeloid lineage cells to promote their survival, proliferation, and differentiation (reviewed in Bagley et al., 1997). The GM-CSF receptor includes α and β subunits, which heterodimerize to form an active signaling complex that activates downstream effectors including the Ras-Raf-MAP kinase and JAK2-STAT5 cascades (Bagley et al., 1997; Doyle and Gasson, 1998). The receptors for GM-CSF, interleukin 3 (IL-3), and interleukin 5 (IL-5) share a common β subunit (β^c) that associates with unique α chains that confer high-affinity binding for the respective cytokines (reviewed in Bagley et al., 1997). JMML cells selectively form excessive numbers of colony-forming unit granulocyte-macrophage (CFU-GM) progenitor colonies in methylcellulose cultures stimulated with low concentrations of GM-CSF (Emanuel et al., 1991, 1996). Murine *Nf1*-deficient fetal hematopoietic cells are also hypersensitive to GM-CSF in vitro (Bollag et al., 1996; Largaespada et al., 1996); this observation directly links the cellular phenotype with inactivation of *Nf1*. While these data raise the possibility that GM-CSF hypersensitivity contributes to leukemic growth in JMML (Emanuel et al., 1991, 1996; Arico et al., 1997), hematopoietic

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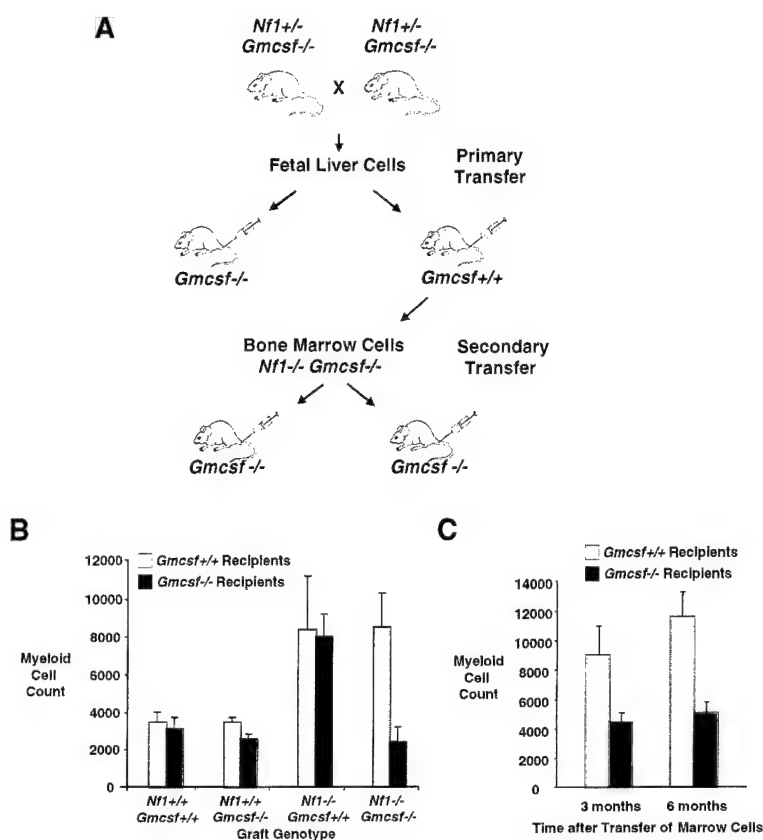


Figure 1. The Influence of *Nf1* and *Gmcsf* Genotypes on Myeloid Cell Counts in Primary and Secondary Recipients

(A) Overview of adoptive transfer studies. *Nf1*^{+/-} *Gmcsf*^{-/-} mice were intercrossed to produce *Nf1*^{+/+} *Gmcsf*^{-/-} and *Nf1*^{-/-} *Gmcsf*^{-/-} fetal liver cells for transfer into wild-type or *Gmcsf*^{-/-} hosts. Secondary transfers were performed using bone marrow cells from wild-type recipients that had previously been repopulated with *Nf1*^{-/-} *Gmcsf*^{-/-} cells and had clinical evidence of MPD.

(B) Myeloid cell counts (\pm SEM) 3 months after adoptive transfer in mice engrafted with donor fetal liver cells ($n = 75$). *Gmcsf*^{-/-} recipients of *Nf1*^{-/-} *Gmcsf*^{-/-} grafts showed lower myeloid counts than recipients in which either host ($p < 0.01$) or graft ($p < 0.005$) could produce GM-CSF.

(C) Ten pairs of irradiated secondary wild-type (open bars) and *Gmcsf*^{-/-} (closed bars) recipients received the same bone marrow cells. Myeloid cell counts (\pm SEM) were elevated in wild-type versus *Gmcsf*^{-/-} hosts 3 and 6 months later ($p < 0.05$ and $p < 0.005$, respectively). The myeloid counts of the *Gmcsf*^{-/-} recipients are in the normal range.

growth factors and their receptors demonstrate substantial structural, biochemical, and functional redundancies. Many activated cytokine receptors, including those for GM-CSF, IL-3, and IL-5, increase Ras-GTP levels by stimulating nucleotide exchange on Ras (Sato et al., 1991). Indeed, *Nf1*-deficient bone marrow cells hyperactivate MAP kinase in response to IL-3 and stem cell factor (SCF) as well as GM-CSF and demonstrate excessive myeloid progenitor colony growth in response to a combination of IL-3 and SCF (Zhang et al., 1998). Thus, it is uncertain whether GM-CSF plays an integral role in myeloid leukemogenesis or is only one of multiple growth factors that stimulate abnormal growth of *Nf1*-deficient cells.

Gmcsf-deficient mice are viable and display normal hematopoiesis (Dranoff et al., 1994). Bone marrow or fetal liver cells from these animals can repopulate irradiated wild-type and *Gmcsf*^{-/-} recipients. Similarly, homozygous inactivation of β^c is not associated with significant hematopoietic defects (Nishinakamura et al., 1995; Robb et al., 1995). The availability of *Gmcsf* mutant mice allowed us to test whether physiologic levels of GM-CSF are essential to drive the excessive growth of *Nf1*-deficient myeloid lineage cells in vivo. Here we show that the absence of GM-CSF markedly attenuates the MPD that arises in irradiated mice after adoptive transfer of *Nf1*^{-/-} fetal liver cells. We further find that GM-CSF remains essential to maintain this MPD after it is established and that *Nf1*-deficient cells are hypersensitive to exogenous recombinant GM-CSF in vivo. These data underscore the specificity of individual growth factors

in determining cellular phenotypes and have implications for devising better therapies for JMML patients.

Results

Gmcsf and *Nf1* mutant mice were generated from 129/Sv embryonic stem (ES) cells (Dranoff et al., 1994; Jacks et al., 1994). We initiated these studies with 129/Sv *Nf1* mice and *Gmcsf* mice in which the disrupted allele had been crossed into a BALB/c background for four generations. *Nf1* and *Gmcsf* have been mapped 17 cM apart on chromosome 11 (Jackson Lab Internet Site, April, 1999: <http://www.informatics.jax.org>). We therefore mated *Gmcsf*^{-/-} and *Nf1*^{+/-} mice to produce *Gmcsf*^{+/-} *Nf1*^{+/-} animals, backcrossed the offspring with the *Gmcsf*^{-/-} line, and screened for recombinants that carried both mutant alleles on the same chromosome. Of 99 *Nf1*^{+/-} pups, 11 founders with the desired *Nf1*^{+/-} *Gmcsf*^{-/-} genotype were identified. *Nf1*^{+/-} *Gmcsf*^{-/-} mice were mated to produce E13.5 fetal liver cells for adoptive transfer into irradiated *Gmcsf*^{-/-} or wild-type hosts (Figure 1A). These cells were injected into the tail veins of *Gmcsf*^{-/-} or wild-type recipients from a BALB/c or a mixed BALB/c \times 129/Sv background that had been conditioned with 8.5 Gy (850 rads) of fractionated total body irradiation. This dose was lethal in 100% of BALB/c mice that did not receive a source of stem cells. Unexpectedly, only 6 of 32 *Gmcsf*^{-/-} recipients injected with *Nf1*^{-/-} *Gmcsf*^{-/-} fetal liver cells were engrafted with donor cells. Engraftment rates were higher in the other host/donor cell genotype combinations; however, the

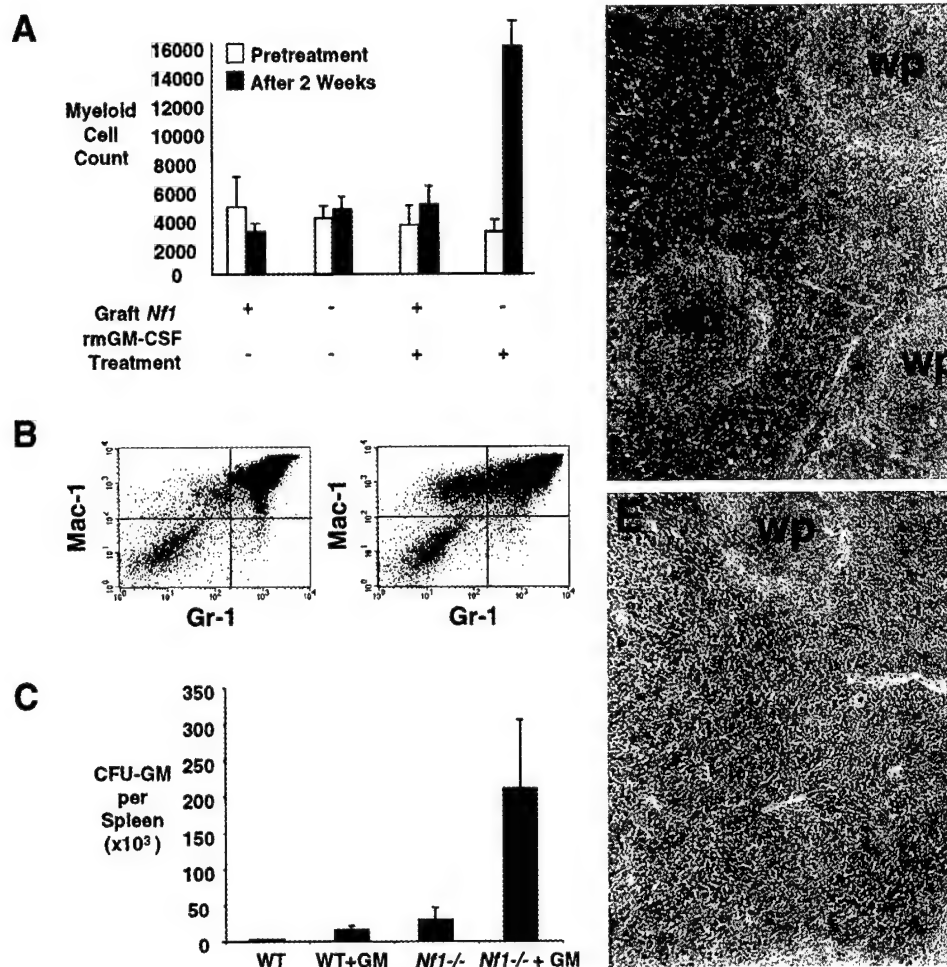


Figure 2. Recombinant GM-CSF Treatment of Secondary Recipients

(A) Myeloid cell counts (\pm SEM) in rmGM-CSF-treated and untreated recipients prior to and after 2 weeks of treatment with rmGM-CSF. Recipients of *Nf1*^{-/-} *Gmcsf*^{-/-} grafts that received rmGM-CSF had elevated myeloid counts when compared to all controls ($p < 0.01$).

(B) Mac-1 and Gr-1 expression on bone marrow mononuclear cells isolated from secondary *Gmcsf*^{-/-} recipients engrafted with *Nf1*^{-/-} *Gmcsf*^{-/-} cells. The left panel shows FACS data from an untreated mouse, and the right panel is from a recipient that received GM-CSF. The percentage of Mac-1⁺ Gr-1^{lo} cells is markedly increased in the GM-CSF-treated animal.

(C) Numbers of CFU-GM in the spleens in rmGM-CSF-treated and control mice (\pm SEM) sacrificed after 2 weeks. Recipients of *Nf1*^{-/-} *Gmcsf*^{-/-} cells that received rmGM-CSF showed a marked expansion of CFU-GM in the spleen.

(D and E) Low power (5 \times objective magnification) view of spleen sections from *Gmcsf*^{-/-} recipients engrafted with *Nf1*^{-/-} *Gmcsf*^{-/-} cells. (D) is from an untreated mouse and shows a normal distribution of red pulp and white pulp (wp). In contrast, the splenic sections from rmGM-CSF mice showed expansion of the red pulp with loss of normal architecture (E).

mixed genetic backgrounds preclude any definitive conclusions regarding the effects of *Nf1* and *Gmcsf* on the repopulating potential of fetal liver cells.

Recipient mice were monitored to determine if the inability of graft cells, host stroma, or both to produce GM-CSF modified the phenotype of the MPD that follows adoptive transfer of *Nf1*^{-/-} cells. This disorder is characterized by elevated myeloid blood cell counts (neutrophils and monocytes) with infiltration of the spleen (Largaespada et al., 1996). Myeloid cell counts (Figure 1B) and spleen sizes were normal in the recipients of wild-type or heterozygous *Nf1* fetal liver cells irrespective of the *Gmcsf* genotypes of either donor cells or hosts. Spleen sections revealed no myeloid infiltrates. Elevated leukocyte counts and extensive splenic infiltration were observed in wild-type recipients of either

Nf1^{-/-} *Gmcsf*^{-/-} or *Nf1*^{-/-} *Gmcsf*^{+/-} fetal liver cells as well as in *Gmcsf*^{-/-} mice that were injected with *Nf1*^{-/-} *Gmcsf*^{+/-} cells (Figure 1B). Thus, GM-CSF production by either the host or the graft is sufficient to induce MPD.

By contrast, myeloid counts in the blood of the six *Gmcsf*^{-/-} recipients successfully engrafted with donor *Nf1*^{-/-} *Gmcsf*^{-/-} fetal liver cells were in the same range as in recipients that received wild-type *Nf1* cells 3 months after adoptive transfer (Figure 1B). Four of these mice survived to 6 months, one of which developed leukocytosis with an elevated myeloid cell count between 3 and 6 months. By 9 months post transplant, 3 of the 4 mice had elevated myeloid counts and splenomegaly that were associated with extensive myeloid cell infiltration at necropsy.

We next isolated bone marrow cells from three wild-type recipients more than 6 months after transplant and transferred them into ten pairs of wild-type and *Gmcsf*^{-/-} hosts as outlined in Figure 1A. These marrow donors had characteristic features of the JMML-like MPD, including leukocytosis and splenomegaly. All 20 secondary recipients survived with engraftment of the *Nf1*^{-/-} *Gmcsf*^{-/-} donor cells. *Gmcsf*^{-/-} recipients had significantly lower myeloid counts than wild-type mice injected with the same bone marrow cells 3 and 6 months after adoptive transfer (Figure 1C). Together the primary and secondary transplant data indicate that GM-CSF is involved in both initiating and maintaining the overproliferation of *Nf1*-deficient myeloid cells in vivo. However, this cytokine is not absolutely essential, as some recipients of *Nf1*^{-/-} cells developed MPD with prolonged latency in the absence of GM-CSF.

Eight *Gmcsf*^{-/-} recipients of secondary bone marrow transplants were randomly assigned to receive treatment with recombinant murine GM-CSF (rmGM-CSF) or to observation only beginning 3 months after adoptive transfer. As an additional control, four *Gmcsf*^{-/-} hosts engrafted with *Nf1*^{+/+} *Gmcsf*^{-/-} cells received rmGM-CSF, and four other mice were observed. Treatment consisted of twice daily intraperitoneal injections of 50 ng of rmGM-CSF for one week, followed by 100 ng of rmGM-CSF in the second week. These doses do not induce leukocytosis in wild-type mice (Metcalf et al., 1987). Myeloid cell counts were similar in all four groups at baseline (Figure 2A) and after one week of rmGM-CSF treatment (data not shown). However, all of the recipients engrafted with *Nf1*^{-/-} *Gmcsf*^{-/-} cells that received rmGM-CSF developed markedly elevated myeloid counts by the end of the second week, while these counts were unchanged in the other cohorts (Figure 2A). Fluorescent-activated cell sorting (FACS) analysis revealed an expanded population of immature myeloid (Mac-1⁺/Gr-1⁺) cells in the bone marrows of the treated *Nf1*^{-/-} *Gmcsf*^{-/-} mice (Figure 2B). Treatment with rmGM-CSF also resulted in a marked expansion of CFU-GM in the spleens of *Nf1*^{-/-} *Gmcsf*^{-/-} recipients (Figure 2C). Splenomegaly was only detected in mice engrafted with *Nf1*^{-/-} cells that had received rmGM-CSF; pathologic sections revealed extensive myeloid infiltration and disruption of splenic architecture (Figures 2D and 2E). Thus, doses of recombinant murine GM-CSF that had no effects in mice reconstituted with wild-type hematopoietic cells rapidly induced MPD in recipients engrafted with *Nf1*-deficient cells.

Discussion

We have shown that GM-CSF plays a central role in establishing and maintaining the MPD that emerges when *Nf1*^{-/-} hematopoietic cells are transferred into irradiated recipients. These data implicate a selective inability to downregulate Ras-GTP in response to GM-CSF in the pathogenesis of JMML, a disease that shares genetic, biochemical, and clinical features with the murine MPD (Figure 3). The specificity of this effect is surprising because of the substantial functional redundancy between different hematopoietic growth factors

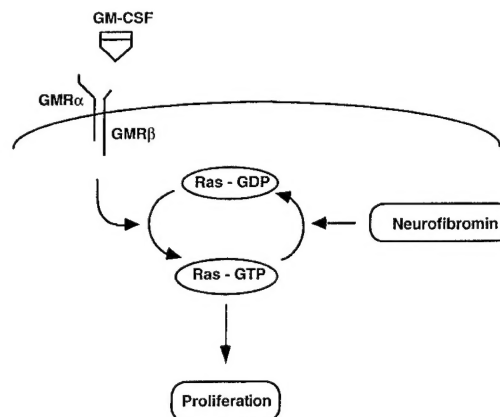


Figure 3. A Model for Ras, Neurofibromin, and GM-CSF Interactions in Myeloid Growth Control

The level of Ras activation induced by GM-CSF has a profound impact on myeloid cell fate in vivo. Because neurofibromin is required to downregulate growth-promoting signals transduced from the GM-CSF receptor, wild-type recipients of *Nf1*^{-/-} fetal liver cells develop MPD. However, the penetrance of the MPD phenotype is markedly attenuated in the absence of GM-CSF. These data indicate GM-CSF, Ras, and neurofibromin as components of a linear signaling pathway that induces leukemic growth when it is deregulated.

and their receptors. Although *Nf1*-deficient hematopoietic cells demonstrate hyperactive Ras-MAP kinase signaling and excessive in vitro progenitor colony growth in response to IL-3 and SCF (Zhang et al., 1998), these growth factors cannot fully substitute for GM-CSF in vivo. Other cell culture experiments have implicated interleukin 1 β and tumor necrosis factor α in the pathologic growth of JMML cells (Emanuel et al., 1996; Arico et al., 1997). While our data do not exclude a secondary role for these and other cytokines in JMML, they provide strong evidence that GM-CSF hypersensitivity is central to the disease phenotype.

Previous studies have implicated elevated levels of GM-CSF and deregulated GM-CSF receptor signaling in aberrant myeloid growth. For example, transferring bone marrow cells engineered to overexpress GM-CSF into recipient mice induces a fatal myeloid disorder with some features of JMML (Johnson et al., 1989), and a strain of GM-CSF transgenic mice demonstrates excessive proliferation of myelomonocytic cells with tissue infiltration (Lang et al., 1987). In addition, mice transgenic for an activated mutant allele of β^c consistently develop erythrocytosis, granulocytic hyperplasia, and splenomegaly (D'Andrea et al., 1998). Our data extend these reports by showing that physiologic levels of GM-CSF can drive excessive proliferation in a model that recapitulates the genetic and biochemical alterations of JMML.

Studies comparing GM-CSF and IL-3 signaling in transfected FDCP cells implicate the respective α chains as important in specifying downstream targets and in regulating the balance between proliferation and differentiation (Evans et al., 1999). Intriguing features specific to the GM-CSF receptor include the existence of preformed $\alpha\beta$ complexes within the plasma membrane, and the observation that GM-CSF binding approaches equilibrium faster than IL-3 or IL-5 with a fraction of GM-CSF

receptors showing rapid binding kinetics (Woodcock et al., 1997). Furthermore, the pool of preformed GM-CSF receptors may participate in signaling in the absence of GM-CSF by being recruited to activated IL-3 and IL-5 receptors (Woodcock et al., 1997). Bagley et al. (1997) have suggested that preformed GM-CSF receptors normally provide a weak constitutive survival signal in hematopoietic cells in the absence of ligand binding. Inactivation of *Nf1* might amplify this signal and thereby reduce the amount of cytokine required to trigger proliferation. Some *Gmcsf*^{-/-} recipients engrafted with *Nf1*^{-/-} *Gmcsf*^{-/-} fetal liver cells ultimately developed MPD. SCF, IL-3, or another hematopoietic growth factor that hyperactivates Ras signaling in *Nf1*-deficient cells may contribute to clonal outgrowth in the absence of GM-CSF by a direct mechanism or indirectly by increasing the likelihood of acquiring somatic mutations. Alternatively, constitutive signaling from GM-CSF receptor $\alpha\beta$ heterodimers that form in the absence of ligand or their recruitment to activated IL-3 receptor complexes could account for the delayed onset of MPD in the absence of GM-CSF.

Continuous expression of an oncogenic *RAS* allele was required both to induce tumor formation and to sustain the growth of established cancers in a murine melanoma model that involves multiple genetic lesions (Chin et al., 1999). Similarly, Felsher et al. (1999) found that persistent *MYC* overexpression was essential to maintain lymphoma growth in transgenic mice (Felsher and Bishop, 1999). Inactivation of *NF1* is strongly implicated in the development of JMML (Shannon et al., 1994; Miles et al., 1996; Side et al., 1997), and we have now shown that *Nf1*-deficient cells from mice with established MPD remain dependent upon GM-CSF for abnormal growth. GM-CSF receptor antagonists have been proposed as potential therapeutics for JMML (Iversen et al., 1996, 1997; Frankel et al., 1998). JMML cells exposed to a peptide antagonist of the GM-CSF receptor selectively undergo apoptosis in vitro (Iversen et al., 1996), and this agent has also shown efficacy in a pre-clinical xenograft model (Iversen et al., 1997). Our findings support this approach; however, the observation that *Nf1*-deficient fetal liver cells are capable of producing MPD in the absence of GM-CSF suggests that receptor antagonists might not be curative unless they are combined with other modalities. In addition, although recombinant GM-CSF is used frequently in clinical transplant protocols to facilitate hematopoietic recovery (Nemunaitis and Singer, 1993), our data argue strongly that it should not be given to children with JMML as it will likely support the survival and proliferation of residual leukemic cells.

Experimental Procedures

Mice

Nf1 and *Gmcsf* mutant mice are described elsewhere (Dranoff et al., 1994; Jacks et al., 1994). Wild-type 129/Sv and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice were housed in the UCSF Animal Care Facility and were fed pelleted chow and acidified water ad libitum. The UCSF Committee for Animal Research approved the experimental procedures.

Isolation of Hematopoietic Cells and Genotyping

Nf1^{+/-} *Gmcsf*^{-/-} or *Nf1*^{+/-} *Gmcsf*^{+/-} mice were mated to produce embryos of all combinations of *Nf1* and *Gmcsf* genotypes. Single-cell suspensions of E13.5 fetal liver cells and bone marrow mononuclear cells were prepared as described (Zhang et al., 1998). Splenocytes were prepared from transplant recipients by macerating spleens with the butt of a 5 mL syringe and then passing cells through a Falcon 2350 70 μ cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). Hematopoietic cells were kept at 4°C prior to use. Cell viabilities were ascertained by trypan blue exclusion.

The polymerase chain reaction (PCR) was used to determine provisional embryonic *Nf1* and *Gmcsf* genotypes so that fetal liver cell experiments could be performed within 24 hr of harvest. *Nf1* alleles were amplified and resolved as described elsewhere (Zhang et al., 1998). *Gmcsf* genotypes were determined by PCR using a common 5' primer, GGAGCATGAGAGTCACTT, and either a 3' primer, CAGACTGCCTTGGGAAAA, to amplify the targeted allele or a 3' primer, ACTTCTCCCTACTCCCTT, for wild-type *Gmcsf*. Amplification conditions for *Gmcsf* were as described for *Nf1* (Zhang et al., 1998). Southern analysis was performed on tail DNA to determine the genotype of mice used for all experiments and to confirm embryo genotypes. Donor cell engraftment was defined as the presence of a visible donor-specific *Gmcsf* or *Nf1* restriction fragment seen on an autoradiograph of DNA extracted from peripheral blood.

Adoptive Transfer

Recipients were irradiated using a Cs137 source that generates 93 cGy/min. BALB/c and mixed BALB/c-129/Sv recipients received 850 cGy of TBI in fractions of 450 cGy and 400 cGy separated by a period of 6–8 hr. Adoptive transfers were performed essentially as described elsewhere (Mahgoub et al., 1999). For transplants of fetal livers, 2–5 $\times 10^5$ cells suspended in 450 μ L of IMDM/20% FCS were injected. Doses of 4–7 $\times 10^6$ bone marrow cells were used to perform secondary transplants.

Follow-up of Recipients

Mice were bled at 1, 3, 6, and 9 months after transplant by nicking dorsal tail veins. White blood cell counts (WBC) were measured using a Hemavet CDC Mascot cytometer (CDC Technologies, Oxford, CT). Peripheral blood films prepared from each sample were stained with a modified Wright-Giemsa stain. Manual 100-cell leukocyte differentials were performed on each sample. The total myeloid cell count was calculated by multiplying the percentage of myeloid cells by the total WBC count. When mice were either sacrificed or died, spleens were weighed, and histologic sections of marrow, spleen, and liver were prepared.

Treatment with rmGM-CSF

rmGM-CSF (Peprotech, Rocky Hill, NJ) was injected intraperitoneally through a 28-gauge needle in 200 μ L of phosphate-buffered saline containing 1% heat-inactivated FCS twice daily at 8:00 am and 8:00 p.m. Bone marrow mononuclear cells from transplant recipients treated with rmGM-CSF and untreated controls were stained with FITC-conjugated anti-Gr1 antibody and PE-conjugated anti-Mac1 antibody as above. Viable cells were analyzed with a Becton-Dickinson FACScan and CellQuest software.

Statistical Methods

Means of continuously measured values were compared using unpaired two-tailed Student's *t* tests. Proportions in experimental groups were compared using the chi-square test. A *p* value less than 0.05 was accepted as statistically significant.

Acknowledgments

This work was supported, in part, by NIH grant CA72614 to K. M. S., by American Cancer Society grant DB80030 to K. M. S. and D. W. C., and by NIH grant CA74886 to G. D. Additional funding support was provided by the Frank A. Campini Foundation (K. M. S.), the Herman W. Wells Association (D. W. C.), and the Cancer Research Institute/Partridge Foundation (G. D.). T. J. is an Associate Investigator of the Howard Hughes Medical Institute (HHMI), and R. A. B. was an HHMI Medical Student Research Fellow. We are indebted to Brigit

Taylor for technical advice, and to Bruce Blazar, Jason Cyster, James Downing, Nigel Killeen, and Catherine Verfaillie for helpful suggestions.

Received October 18, 1999; revised November 15, 1999.

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